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(54) Title A method for the selective immunological determination of intact procollagen peptide (Type III) and procollagen (Type III) in body fluids and means for carrying it out

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Specification

A method for the selective immunological determination of intact procollagen peptide (type III) and procollagen (type III) in body fluids, and means for carrying it out

Procollagen peptide (type III) is the amino-terminal propeptide of collagen (type III), which is cleaved off outside the cell after secretion of the procollagen (type III) molecule. The concentration of this procollagen peptide in body fluids can be determined using a radioimmunological determination method as described in European Patent No. 4940. Knowledge of the serum concentration of the peptide allows conclusions to be drawn about the activity of fibrotic disorders, such as; for example, of the liver [Rohde, H. et al. Eur. J. Clin. Invest. 9, 451 - 459 (1979)].

However, accurate selective determination of procollagen peptide (type III) in serum and other body fluids is not possible using the methods hitherto described, because the polyclonal antibodies which are used in these methods react, with different, lower affinity, with various antigens which occur in serum and some of which are breakdown products of procollagen peptide (type III) [Niemela, O. et al. Clin. Chim. Acta 124, 39 - 44 (1982)]. The result of this is that the serum dilution plots and the dilution plots of other body fluids are not parallel to the calibration plot constructed using pure procollagen peptide (type III), and hence it is necessary to determine the antigen content in several dilutions of each unknown sample in order to establish the antigen concentration via the 50 % intercept on the dilution plot.

Another disadvantage of these methods is that, because the cross-reactivity of the antibodies with rat or mouse antigen is too low, no determination of the antigen concentration

in body fluids from these species has been possible.

It is possible to determine the concentrations of the antigens in rat serum using the method described by Schuppan et al. [J. Hepatol. 3, 27-37 (1986)]. However, this method has the same disadvantage as that described for the method for human sera [Niemelä et al. Clin. Chim. Acta 124, 39 - 44 (1982)] that the inhibition plots are not parallel for different body fluids.

This technical problem can be solved using the method of European Patent Application 0,089,008, in which the antibodies used have comparable affinities for intact procollagen peptide (type III) and its breakdown product Col 1. This method determines intact and degraded procollagen peptide (type III) together, but this results in imprecision in the diagnostic conclusions because the normal popuulation and the patient population may overlap greatly.

It has now been found, surprisingly, that when a peptide with a defined amino acid sequence is used for immunization it is possible to obtain antibodies with whose aid specific determination of intact procollagen peptide (type III) is possible. Moreover, surprisingly, using these antibodies it is possible to determine the procollagen peptide (type III) content in body fluids of rats or mice, which is of use for examining fibrosuppressive substances in animal experiments.

Thus the invention relates to a method for the immunological determination of amino-terminal procollagen peptide (type III) using antibodies, which comprises

a) immunization of animals with a peptide of the sequence

I-C-E-S-C-P-T-G-G-Q-N-Y-S-P,

bound to an immunogenic protein,

b) obtaining from the serum the antibodies which react with intact amino-terminal procollagen peptide (type III) and

c) determination of the amount of amino-terminal procollagen peptide (type III) and of procollagen (type III) via the antigen-antibody complex which is formed.

The invention also relates to antibodies which are raised by immunization of animals with the abovementioned peptide which is bound to an immunogenic protein.

The invention is described in detail hereinafter, especially in its preferred embodiments. The invention is also defined in the claims.

For the preparation of the antibodies, animals, preferably rodents such as, for example, rabbits or guinea pigs, or goats and sheep are immunized with the peptide of the sequence

I-C-E-S-C-P-T-G-G-Q-N-Y-S-P

coupled to a suitable immunogenic protein in the presence of complete adjuvant. Rabbits are particularly preferably used. The immune response is enhanced by repeated booster injections, for example at intervals of 4 to 8 weeks. The success of immunization is checked by determining the concentration of antibodies in a radioimmunological binding assay [R. Timpl and L. Risteli, Immunochemistry of the extracellular matrix, H. Furthmayr Ed., Vol. 1, 199 (1982)].

All immunogenic proteins are suitable as proteins to which the said peptide can be bound. Hemocyanin, albumin or polylysine are preferably used. The peptide can be prepared by methods known to the expert, as described by,

for example, G. Barany and A.B. Merrifield in The Peptides Vol. 2, pp 3-254 (1980), Academic Press, or E. Brown, R.C. Sheppard and B.J. Williams, J. Chem. Soc. Perkin Transact. 1, 1161 (1983).

The antibodies according to the invention can be used as serum or purified in various immunological methods, including all types of radioimmunoassay, for example sequential saturation analysis or equilibrium analysis, labeling with chloramine T or Bolton-Hunter reagents [Felber, Meth. Biochem. Anal. 22, 1 (1974); Shelley et al., Clin. Chem. 19, 146 (1975)] as well as in other competitive binding assays such as fluorescence or enzyme immunoassays, chemiluminescence or other immunoassays. The antibodies can thus be used in immunological methods for the isolation and characterization and for the quantitative determination of procollagen peptide (type III) in tissues and body fluids. The procedure for quantitative determination makes use of methods known to the expert, by reacting a liquid sample which contains procollagen peptide (type III) with the antibodies according to the invention, and determining the amount of procollagen peptide (type III) via the antigen-antibody complex which is formed. It is of no consequence for this whether the procollagen peptide (type III) is still linked to the amino terminus of procollagen (type III) or not. The degradation products of procollagen peptide (type III), especially Col 1, which have hitherto interfered in the immunological determination, are not among the species detected by the antibodies according to the invention.

The invention is explained further in the examples which follow. Unless otherwise indicated, percentage data relate to weight.

Example 1

Preparation of the peptide/hemocyanin conjugate compound

100 mg of hemocyanin (dialyzed against 5 x 3 l of water and then freeze-dried) are dissolved in 3 ml of water, and 24 mg of the peptide I-C-E-S-C-P-T-G-G-Q-N-Y-S-P are added. While stirring at room temperature, a total of 1 g of N-cyclohexyl-N'-[2-(4-morpholinyl)ethyl]carbodiimide-methyl-p-toluenesulfonate is added in portions over the course of 2 hours. The reaction mixture is stirred overnight and then dialyzed against a total of 5 x 10 l of water for 24 hours, and the product is subsequently isolated by freeze-drying. 137 mg of the conjugate compound are obtained.

Example 2

Radioimmunological binding assay

300 µl of antiserum from rabbits immunized with the conjugate prepared as in Example 1 are, in a suitable dilution, incubated overnight with 100 µl of a procollagen peptide type III solution (1 ng of protein/100 µl, prepared as described in European Patent 4940, Example 1) which is radioactively labeled with ^{125}I. The antigen-antibody complexes which are formed are precipitated by addition of an antiserum which is directed against immunoglobulin G of the species used for the immunization and is from a different species. Following centrifugation and decantation of the supernatant, the amount of precipitated radioactivity is determined in a γ-scintillation spectrometer.

Example 3

Radioimmunoassay

0.2 ml of the sample which is to be analyzed or of pro-

collagen peptide (type III) standard is incubated at $4^{o}C$ overnight with an amount of antiserum (in 0.1 ml of buffer) which is limiting with respect to the amount of labeled antigen. Addition of 0.1 ml of ^{125}I-labeled procollagen peptide (type III) (contains 1 ng of protein) is followed by incubation at $4^{o}C$ for 6 - 8 hours. The antigen-antibody complexes which are formed are precipitated by use of an antiserum directed against IgG of the species used for the immunization. Centrifugation and decantation of the supernatant are followed by determination of the precipitated radioactivity in a γ-scintillation spectrometer.

It is then possible, by comparison with a calibration plot which has been constructed by using standards with different concentrations of procollagen peptide (type III), to determine the concentration of procollagen peptide (type III) in the unknown solution.

Example 4

Determination of the molecular weight distribution of the antigens which react with the antibodies according to the invention and are present in the serum of, for example, cattle and hogs

1 ml of serum is fractionated by gel filtration chromatography on a Sephacryl(R) S 300 column (1.6 x 130 cm) equilibrated in phosphate-buffered saline, PBS, containing 0.04 % Tween 20. 0.2 ml of each of the fractions (2.8 ml each) is used in the radioimmunoassay of Example 3.

Fig. 1a shows the elution profile of the antigen from porcine serum, and Fig. 1b shows that of the antigen from bovine serum, comparing with the profile of the antigen determined as in European Patent 4940. Peaks 1/1a correspond to intact procollagen type III and pN collagen type III (procollagen lacking the C-terminal end); peaks 2/2a correspond to intact amino-terminal procollagen peptide

type III; peaks 3/3a correspond to Col 1 and breakdown products of amino-terminal procollagen peptide type III with the same molecular weight as Col 1.

Patent Claims: HOE 8 /F 130

1. A method for the immunological determination of amino-terminal procollagen peptide (type III) using antibodies, which comprises
a) immunization of animals with a peptide of the sequence

I-C-E-S-C-P-T-G-G-Q-N-Y-S-P

bound to an immunogenic protein,

b) obtaining from the serum the antibodies which react with intact amino-terminal procollagen peptide (type III) and

c) determination of the amount of amino-terminal procollagen peptide (type III) and of procollagen (type III) via the antigen-antibody complex which is formed.

2. The method as claimed in claim 1, wherein the peptide is bound to hemocyanin, albumin or polylysine.

3. The method as claimed in claim 1 or 2, wherein rodents or goats or sheep are immunized.

4. The method as claimed in claim 3, wherein rabbits are immunized.

5. Antibodies obtainable by immunization of animals with the peptide of the sequence

I-C-E-S-C-P-T-G-G-Q-N-Y-S-P

bound to an immunogenic protein.

6. Antibodies as claimed in claim 5, wherein the peptide is bound to hemocyanin, albumin or polylysine.

7. Antibodies as claimed in claim 5 or 6, wherein rodents or goats or sheep are immunized.

8. Antibodies as claimed in claim 7, wherein rabbits are immunized.

9. A compound composed of the peptide of the sequence

I-C-E-S-C-P-T-G-G-Q-N-Y-S-P

bound to an immunogenic protein.

10. The use of the compound claimed in claim 9 as an antigen.

11. A method according to Claim 1 for the immunological determination of amino-terminal procollagen peptide (type III) using antibodies, substantially as hereinbefore described and exemplified.

12. An antibody according to Claim 5, substantially as hereinbefore described and exemplified.

13. A compound according to Claim 9, substantially as hereinbefore described and exemplified.

14. Use according to Claim 10, substantially as hereinbefore described and exemplified.

Dated this the 29th day of April, 1988

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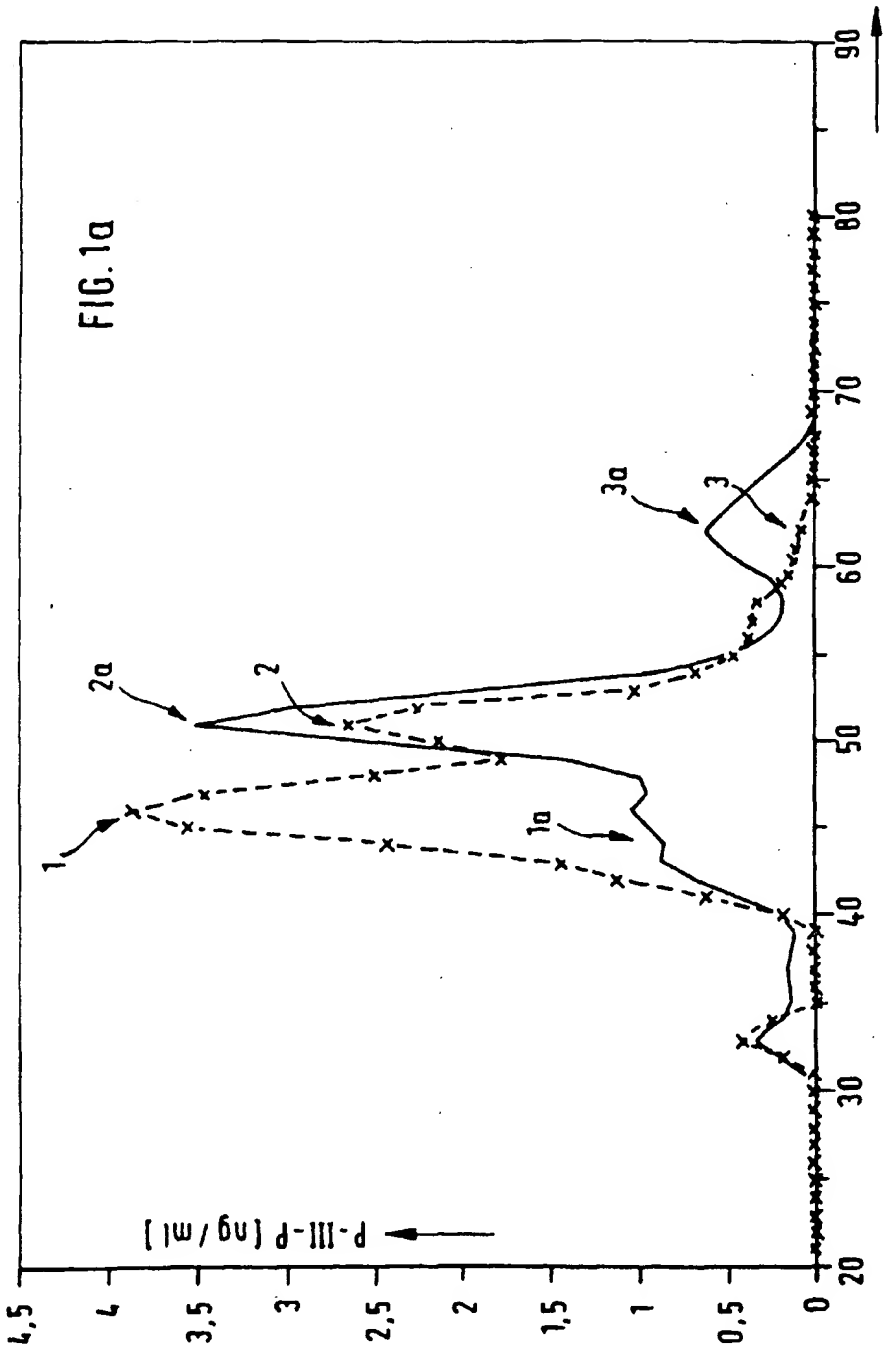

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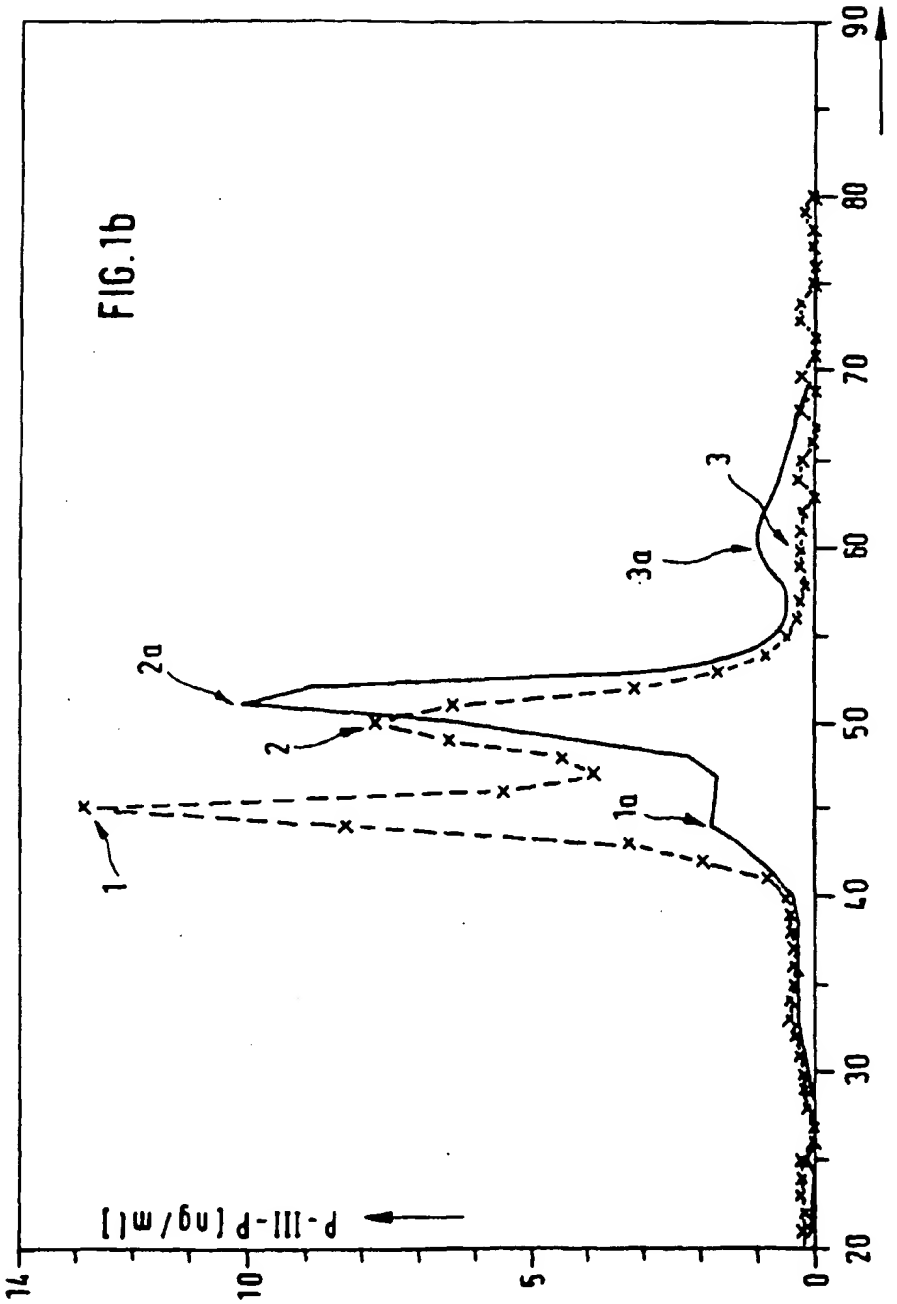

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